UNIVERSITY OF PENNSYLVANIA

A METHOD FOR THE STUDY OF BACTERIAL LIPASE

WALTER LORD OBOLD

A THESIS

IN BACTERIOLOGY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
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The lipolytic activity of bacteria has been demonstrated by various methods. Eijkman[1] made use of a very thin layer of tallow on the bottom of a sterile Petri dish. This was prepared by pouring melted tallow into the dish and quickly draining off the excess fat. Nutrient agar was melted and after it had cooled sufficiently it was inoculated with the microorganism and poured over the fat layer where it was allowed to solidify before incubation. Lipolytic activity was detected by the translucent areas in the opacity of the tallow around the colonies.

Buchanan[2] recognized the activity of extracellular lipase on agar plates containing emulsified fat by the clearing of the medium immediately surrounding the colonies. Hydrolysis of the fat into fatty acids and glycerin presumably accounted for these clear zones.

Waksman and Davison[3] detected lipoytic activity by observing an increase in acidity as shown by an indicator such as litmus or brom-cresol-purple. The enzyme was placed in a fat emulsion and incubated at 34° C. for two hours or longer. A boiled enzyme control was treated in the same manner to insure that the acidity was due to the fatty acids liberated by enzyme activity.

Turner[4, 5] used nile blue sulphate in a cottonseed oil beef infusion agar to indicate lipolysis of the oil under anaerobic conditions. In this study Turner's test was found difficult to duplicate and the dye inhibited some bacteria.

Wells and Corper[6] demonstrated lipolytic activity by means of the increased acidity produced by large quantities of dead bacteria on lipide substrates. The suspensions of microorganisms were killed with toluene before the addition of the lipide. The increase in acidity was titrated daily, using phenolphthalein as the indicator.

Recently Berry[7] used the copper soap method for the detection of microbial lipase in living cultures. The medium used was a plain nutrient agar in which melted butter was emulsified. The

microorganism was inoculated onto the agar and after forty-eight hours incubation the plate was flooded for ten minutes with a saturated aqueous solution of copper sulphate and rinsed with water. The colonies producing a lipase showed conspicuous blue-green streaks of insoluble copper soaps.

None of these methods show differences in the activity of the lipases produced by various bacteria. Bacteria with a feeble lipolytic activity were not readily recognized, so a method was developed for the quantitative as well as the qualitative recognition of the lipolytic activity of living bacteria. It requires the use of the ordinary culture and a fat emulsion easily prepared. It is adaptable to any lipide and can be used as a routine test to determine the activity of an extracellular lipolytic enzyme in actively growing cultures.

Preparation of Lipide Emulsion

The lipide emulsion was prepared as follows:

Gum Arabic	40 gm.
Tributyrin	30 cc.
Distilled Water	970 cc.

The gum arabic was emulsified in the water by means of heat and constant stirring; tributyrin (or any lipide desired as a substrate) was added and the contents were thoroughly mixed by means of an electric beater. Forty cubic centimeters of the lipide emulsion were placed in 200 cc. Erlenmeyer flasks and sterilized in the autoclave at 15 pounds pressure for twenty minutes. The flasks were allowed to cool to room temperature before use.

Lipides that were solid at room temperature were raised to their respective melting points and the same amount was added in place of the tributyrin.

Control flasks of the gum arabic emulsion without any lipide were prepared and sterilized as above.

The effect of sterilization of the tributyrin emulsion at the above temperature and pressure was tested. Half of the flasks were titrated with n–10th sodium hydroxide for free acid before

sterilization and an aliquot portion titrated after sterilization. The following values show that there was no appreciable effect on tributyrin under these conditions.

Tributyrin Emulsion

cc. n–10th sodium hydroxide required to neutralize the acid

Before Sterilization	*After Sterilization*
1.0	1.0
0.9	1.0
0.9	1.0

The results of three tests are given.

Substrate Used

The following lipides were tested: triacetin, tributyrin, ethyl butyrate, ethyl iso-butyrate, n-butyl n-butyrate, n-amyl n-butyrate, beef fat, lard, crisco, butterine, castor oil, coconut oil, corn oil, cottonseed oil, cod-liver oil, linseed oil, sperm oil, olive oil, butter, and butter fat.

Triacetin was found unsatisfactory due to difficulty of titration and the comparatively rapid rate of hydrolysis in the alkaline medium. Butter fat was prepared by ether extraction and washing with water to remove water-soluble substances. Tributyrin was found most satisfactory for measuring lipolytic activity when compared with the other substrates.

Tributyrin has a molecular weight of 302, boiling point 285° C., a specific gravity of 1.052 and is insoluble in water but very soluble in alcohol and ether. The acidity or pH value in these tests was determined in the water phase. The acidity of the oil phase may be different from this.

Media and Bacteria

It was found that the medium for fat decomposition as used by Rahn[8] was unsatisfactory due to the heavy precipitate of insoluble magnesium phosphate in the presence of ammonium salts.

When following the method of testing described below, plain nutrient broth containing 4 grams of added sodium chloride per liter was found to be satisfactory for determining the presence of lipase in growing cultures of bacteria. The medium was prepared and sterilized in the autoclave in the usual manner. While other modified salt media can be used it was deemed advisable to use the routine broth as prepared in the laboratory and to adopt this method for quantitative study.

The broth after inoculation with the microorganism was incubated for 24 hours at 37.5° C. and then added to the lipide emulsion. Cultures of Pseudomonas aeruginosa and Staphylococcus aureus were most active in producing lipase. Other bacteria used were Streptococcus fecalis, Proteus vulgaris, Escherichia coli and Salmonella enteritidis.

Method of Testing

Ten cubic centimeters of a 24-hour nutrient broth culture of the microorganism were carefully poured into the 200 cc. Erlenmeyer flask containing 40 cc. of the sterile lipide emulsion prepared as previously described. Phenol red solution was added and the pH value was adjusted to 8.4 with n–10th sodium hydroxide. A second 24-hour broth culture of the microorganism was carefully poured into a flask containing the same amount of the gum arabic emulsion without the added lipide. This control flask was adjusted to the same pH value as the test flask. The flasks were placed in an incubator at 37.5° C. and adjusted to pH value 8.4 with the alkali solution, at the end of each twenty-four-hour period always recording the volume of alkali needed each time.

Phenol red was selected as the most satisfactory indicator in the tests performed. It was prepared by dissolving 1 gram of the indicator in 57 cc. of n–20th sodium hydroxide solution and adding distilled water to make a volume of 250 cc. This was the stock solution. The indicator for general use was prepared by diluting 25 cc. of the stock solution to 250 cc. with distilled water. This gave a 0.04 per cent concentration of the indicator. Five drops of this indicator solution was used for 10 cc. of the

material tested. The pH range covered by phenol red is from 6.8 to 8.4 and the color change is from yellow to red.

Activity Values

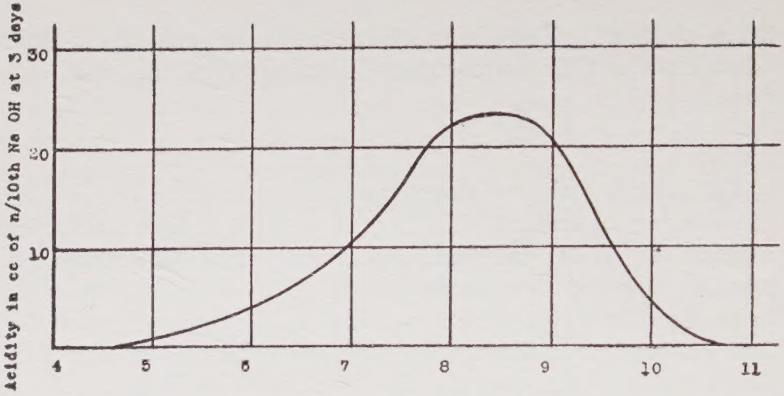
The activity values of the enzyme were calculated by dividing the number of cubic centimeters of n–10th sodium hydroxide required to adjust the test flask to pH value 8.4, minus the number of cubic centimeters of the alkali solution needed to bring the contents of the control flask to the same pH value, by 2 or 3, the time in days. Any activity value greater than 1.00 was typical of the strongly lipolytic bacterial lipases. Such values corresponded to a minimum of 1 cc. of n–10th sodium hydroxide per twenty-four hours in 40 cc. of the emulsion prepared as described.

pH Range

Emulsions of tributyrin were tested to determine the optimum H-ion concentration for the greatest activity of the bacterial lipase. The rate of hydrolysis was determined by daily titration of the increased acidity at each of the pH values selected. The following indicators with their respective pH ranges were used:

Brom-phenol blue	3.0– 4.6
Chlor-phenol red	5.2– 6.8
Brom-thymol blue	6.0– 7.6
Phenol red	6.8– 8.4
Cresol red	7.2– 8.8
Thymol blue	8.0– 9.6
Sulfo orange	11.0–12.6

The optimum pH range for lipolytic activity on tributyrin emulsion at 37.5°C. (Figure 1). was between 7.8 and 9.0. Turner[5] found his micro-organism producing a lipase active over a pH range from 2.0 to 10.0 with an optimum between 6.0 and 7.0. Gortner[9] found an optimum at 8.0 for plant or animal lipase but no action below 5.0.



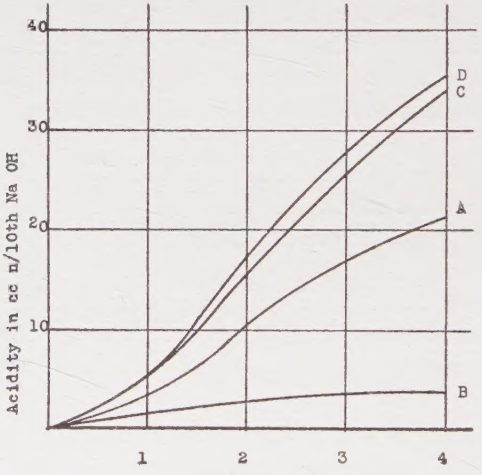
pH VALUE

FIGURE 1

pH RANGE OF LIPASE (P. AERUGINOSA) AT 37.5 C.

Advantage of Emulsification

The degree of dispersion of the tributyrin was more important than the amount of the lipide added. (Figure 2). A simple emul-



DAYS

A— 3 per cent tributyrin in distilled water
B—15 per cent tributyrin in distilled water
C— 3 per cent tributyrin emulsified with gum arabic
D—13 per cent tributyrin emulsified with gum arabic

FIGURE 2

ADVANTAGE OF EMULSIFICATION. (STAPH. AUREUS)

sion was made of 3 per cent and another of 15 per cent tributyrin. The 3 per cent flask showed more activity than the 15 per cent flask when the cultures of bacteria were added and incubated. This increased activity in the flask containing the smaller amount of lipide was presumably due to the slight agitation during titration which facilitated more emulsification in that flask, whereas in the 15 per cent flask the globules were larger and coalesced readily. However when the emulsion was prepared according to the method previously described, there was a marked increase in acidity in both the 3 per cent flask and in the one containing the larger amount of tributyrin. The 3 per cent gum arabic emulsion was used in all routine testing.

Temperature Range

The lipases studied were not easily destroyed by heat. The tests run with heated lipase gave varied results but in no case was the activity as great as in the unheated control. (Figure 3).

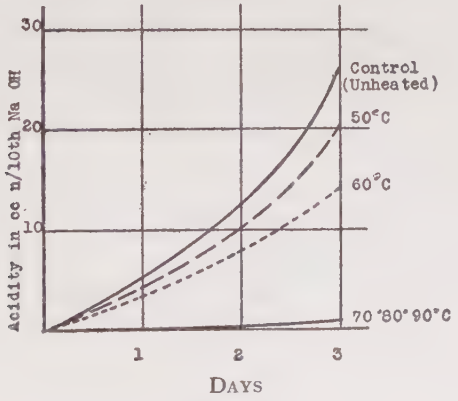


FIGURE 3

EFFECT OF HEATING LIPASE FOR 10 MINUTES AT VARIOUS TEMPERATURES. (P. AERUGINOSA)

The lipolytic activity was not greatly affected by a temperature from 50° to 60° C. for ten minutes but was destroyed at 100° C. in five minutes. In all the tests the enzyme was apparently

reduced in activity as the temperature rose so that in order to destroy the enzyme a definite temperature and time are required.

The incubation temperature of the micro-organism affects the activity of the lipolytic action. (Figure 4). With P. aeruginosa

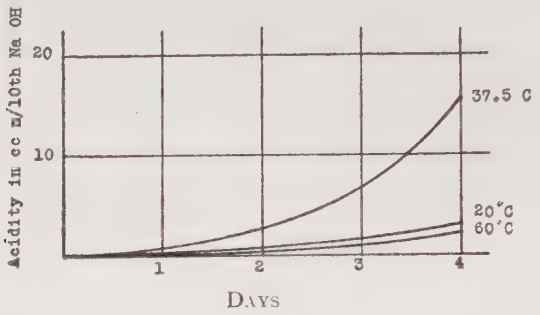


FIGURE 4
EFFECT OF INCUBATION TEMPERATURE. (P. AERUGINOSA)

the optimum incubation temperature for growth gave the greatest acidity in the lipide emulsion.

Bacterial Population

The increase in bacterial population corresponded approximately with the rate of enzyme activity. (Figure 5). The greatest population occurred in four days at 37.5° C. The enzyme activity began to diminish after that time. The bacteria were in their greatest growth rate when they were placed in the lipide emulsion but the rate soon began to fall off as the population was adjusted to the environment.

Comparison with the Copper Soap Method

A comparative study was made between the copper soap method of determining lipolytic activity of micro-organisms as described by Berry[7] and the action of the same culture of micro-organism on an emulsified oil such as tributyrin. It was found impossible to use tributyrin as the lipide in the copper soap method because the copper butyrate was soluble in water. The tributyrin gave a milky opalescence when added to the

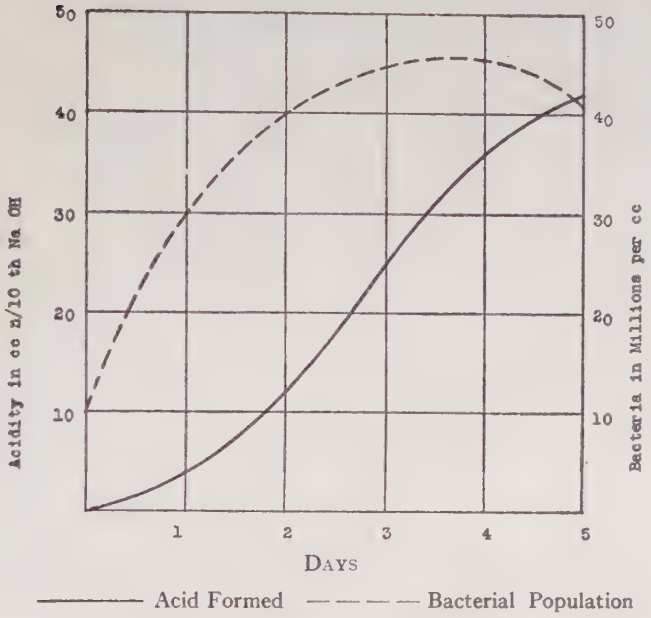


FIGURE 5
LIPASE ACTIVITY AND BACTERIAL POPULATION. (STAPH. AUREUS).

nutrient agar. Microscopic examination showed the agar to be full of small droplets of the oil well dispersed throughout the medium. A culture of P. aeruginosa was grown on an emulsion agar plate. There were definite clear zones around the colonies indicating that the lipide had been hydrolysed. It was noted that this same culture was very active, as shown by the titration values given in Table 1.

This would indicate that the lipase was produced during the growth of the microorganism and was given off as a true extracellular enzyme. This checked with the increase in acidity in the emulsion with increasing periods of time and with the increasing bacterial population. The advantage of titrating the acidity during the growth of the microorganism lies in the fact that although the copper soap method was in part satisfactory from

a qualitative viewpoint, the titration procedure indicated that some bacteria that are negative in that they produce no detectable copper soaps do show some definite hydrolysis of the lipide. The titration method had the advantage in that the results could be expressed numerically, thus offering a quantitative procedure and in that it could be performed with material found in any bacteriological laboratory.

TABLE I
COMPARISON OF COPPER SOAP METHOD WITH TITRATION OF ACID

MICROORGANISM	COPPER SOAP FROM		TITRATION OF TRIBUTYRIN	REMARKS	ACTIVITY VALUE
	BUTTER	BUTTER FAT			
P. aeruginosa "P"	positive	positive	positive	active	1.95
P. aeruginosa "C"	positive	positive	positive	active	1.85
S. aureus "D"	positive	positive	positive	active	1.85
S. aureus "C"	positive	positive	positive	very active	2.45
P. vulgaris	?	negative	negative	feeble	0.65
E. coli	negative	negative	negative	feeble	0.85
E. enteritidis	negative	negative	negative	feeble	0.55
S. fecalis	negative	negative	negative	feeble	0.50
Control	negative	negative	negative	.....	0.30

"P" and "C" refer to different strains of the same species.
?=questionable result.

Discussion

The ability of microorganisms to hydrolyse fats is generally accepted and this hydrolysis is caused by an extracellular lipase which is produced as a result of the normal metabolism of the microorganism. In testing for lipolytic activity the emulsification and stabilization of the lipide was found to be more important than the relative amount of the lipide added. This would indicate that the increase in surface would aid in the detection of the feebly active lipase of some bacteria. The earlier methods do not detect action by bacteria that are only slightly lipolytic, such as E. coli.

Tributyrin was the most satisfactory lipide used in routine testing, although for any particular purpose it is recommended that the individual fat in question be tested, as the lipase may hydrolyse one fat at a much greater rate than another under the

same conditions. Plain nutrient broth was found to be satisfactory for growing the bacteria and for testing for the presence of a lipase. Bacteria vary in the degree of lipolytic activity rather than in the presence of or the absence of the enzyme. It seems probable that all bacteria produce an extracelluar lipase.

The rate of hydrolysis is well established in 48 hours according to repeated tests, so the method described above can be completed in a reasonable period of time. The pH range of lipase activity was from 7.8 to 9.0 with an optimum at 8.2. Temperature affects the activity of the enzyme as it affects the growth of the microorganism. While some lipase withstood 60° C. for 10 minutes and 100° C. for varying periods of time such temperatures are sufficient to reduce the activity markedly. The enzyme activity corresponded directly with the microbial population but was independent of it.

Comparison with the copper soap method showed that some lipolytic activity was not detected by this method as compared with the titration of the increase in acidity in the lipide emulsion. Bacteria that have an activity rate less than 1.00 are not detected by the methods that depend upon the diffusion of the lipase through the agar until it comes in contact with the surface of the oil droplet. E. coli is considered in the literature as a non-lipolytic bacterium, although it produces a lipase with an activity value of about 0.80; whereas P. aeruginosa is detected as a lipolytic bacterium according to earlier methods of testing and it has an activity value of more than 2.0.

By using a wort broth the method described in this paper is adaptable to the study of the lipase produced by yeasts and molds.

Summary

1. Bacteria produce an extracellular lipase that is capable of hydrolysing various lipides when in the emulsoid state.

2. This hydrolysis can be detected both qualitatively and quantitatively by the increase in acidity of the emulsified lipide.

3. Considerable diversity is revealed by various bacteria on the same lipide emulsion.

4. A method has been described for observing and measuring the activity of bacterial lipase.

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